separated from the 4K-PTTHs by gel-filtration through Sephadex G–50 in both *P. c-aureum* and *B. mori*.

Reverse-phase HPLC in the highly purified 4K-PTTH of B. mori (bombixin) and the Polygonia Sephadex G–50/SMPH-active fractions

SMPH-activity of highly purified 4K-PTTH (bombixin) was bioassayed using short-day *Polygonia* pupae. As a result, it was found to show SMPH-activity as strong as the *Polygonia* and *Bombyx* crude SMPH preparations showed (Fig. 3).

In order to separate the SMPH from the 4K-PTTH, the highly purified 4K-PTTH (1000-brain equivalents) was dissolved in 0.2 M ammonium acetate, applied to on reversed-phase column of HPLC (Hi-Pore RP–308) and eluted by a linear gradient of acetonitril concentration of 10–40% in 0.1 M ammonium acetate. The *Polygonia* SMPH-active fractions from Sephadex G–50 were lyophilized and applied to the reversed-phase HPLC in the same manner as above. Sample fractions were divided into two parts, lyophilized, dissolved in distilled water and assayed for SMPH- and 4K-PTTH-activity, respectively.

According to the result of reversed-phase HPLC

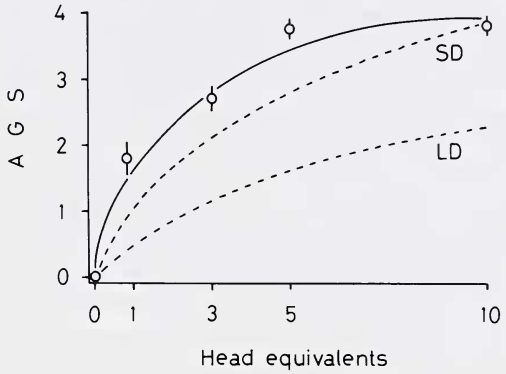

FIG. 3. Dose-dependent response curve of SMPH-activity obtained by highly purified 4K-PTTH of *Bombyx mori* (curved thin line with open circles). Broken lines with letters SD and LD show the does-dependent response curve obtained from the brain-extracts of short-day and long-day pupae [5].

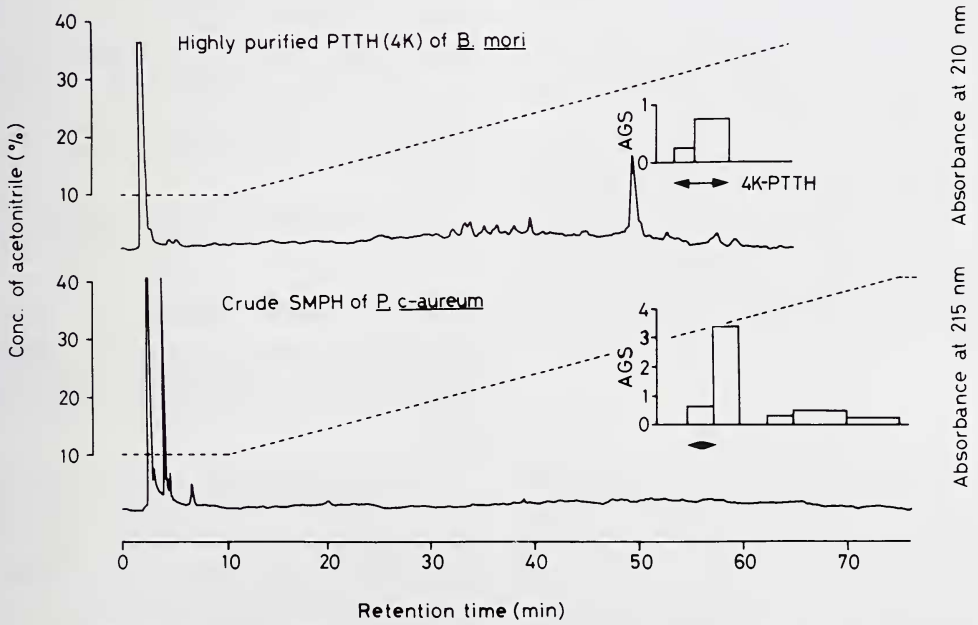

FIG. 4. Reversed-phase HPLC patterns in *Bombyx* highly purified 4K-PTTH (upper panel) and *Polygonia* Sephadex G–50/SMPH-active fractions (lower panel). Solid thin lines show absorbance at 210 nm (upper panel) and 215 nm (lower panel), respectively. Broken lines show the concentration of acetonitril increasing linearly. Histograms show SMPH-activity in the fractions and double-sided arrows indicate 4K-PHHT-active fractions.

of the highly purified *Bombyx* 4K-PTTH, SMPH-activity was detected from two fractions, where two small peaks were detected optically at 210 nm. Furthermore, the two fractions judged as being SMPH-active showed 4K-PTTH-activity by *Papilio* pupal assay (Fig. 4). The fraction showing the highest SMPH-activity was obtained at the acetonitril concentration of 29–34%, which was significantly higher than the concentration at which most 4K-PTTH-active fractions were eluted (28–29%) (Fig. 4: upper panel).

On the other hand, no significant peaks were detected optically at 215 nm by reversed-phase HPLC of *Polygonia* SMPH-active fractions, but 5 fractions showing SMPH-activity were obtained at the acetonitril concentrations of 28–32% and 34–43%. Furthermore, one of the fractions showing SMPH-activity was judged as being 4K-PTTH-active by *Papilio* pupal assay. The *Polygonia* fraction showing the highest SMPH-activity was obtained at the acetonitril concentration of 30–32%, which was almost the same concentration as the *Bombyx* fraction showing the highest SMPH-activity. However, the most active 4K-PTTH fraction was obtained in *P. c-aureum* at the acetonitril concentration of 28–30%, which was a little lower than the concentration of most SMPH-active fractions (30–32%) as observed in the case of the silkmoth, *B. mori,* (Fig. 4: lower panel).

The results indicated that the *Bombyx* and *Polygonia* factors showing SMPH-activity seemed to be similar to those of the 4K-PTTH in the size of its molecules as well as in chemical characteristics. However, the factor showing SMPH-activity did not seem to be identical with the 4K-PTTH in *P. c-aureum*.

DISCUSSION

The mechanism underlying the photoperiodic control of seasonal-morph determination in *Polygonia* involves a neuroendocrine factor producing summer morphs (SMPH) [1–4]. The SMPH can be extracted with 2% NaCl from the brains of *Polygonia* pupae, but is unsuccessful with acetone or 80% ethanol [5]. The SMPH is stable to heating (Table 1), but is thought to be a peptide hormone since it was precipitated by ammonium sulfate (Fig. 1) and inactivated by trypsin-hydrolysis (Table 1). This has been demonstrated in several other neurohormones of insects, i.e., melanization and reddish coloration hormone in *Leucania separata* [8], diapause hormone [9] and PTTHs in *B. mori* [7].

In addition, a factor showing the same activity as *Polygonia* SMPH is present in the brains of the silkmoth, *B. mori* [5]. The *Bombyx* factor could be extracted (with 2% NaCl) and precipitated (by ammonium sulfate at 80% saturation) in almost the same manner as has been demonstrated in the case of 4K- and 22K-PTTHs of *B. mori* [7].

The coincidental behaviors of SMPH and PTTHs can be explained using two hypothetical models, as has been demonstrated in a *Papilio* system underlying the photoperiodic control of seasonal-morph determination [10]. The first model is based on 3 neurohormones, i.e., one SMPH and two PTTHs, and secretion of the SMPH is controlled by larval exposure to appropriate photoperiod and temperature. The coincidental behaviors may be explained, in this model, by a hypothesis that the molecule showing SMPH-activity is similar to those of 4K- and 22K-PTTHs in chemical characteristics in *P. c-aureum.* The second model involves two neurohormones, i.e., one SMPH and one PTTH. One is the SMPH showing 4K-PTTH-activity, whereas the other (22K?) is the true PTTH triggering pupal-adult ecdysis.

From the Sephadex G–50 gel-filtration patterns of SMPH- and 4K-PTTH-activities (Fig. 2), it may be concluded that the molecular sizes of the *Polygonia* and *Bombyx* SMPHs (about 4500) are almost the same as that of the *Bombyx* 4K-PTTH (bombixin: M.W. 4400 [7]) but are far smaller than the size of the *Bombyx* 22K-PTTH (M.W. 22,000 [7]).

We could not provide any evidence with respect to the molecular size of *Polygonia* PTTH triggering pupal-adult ecdysis, but it was thought to be larger than M.W. 15,000. This estimation was based on the number of days required for adult development, i.e., most pupae treated with *Polygonia* Sephadex G–50 fractions emerged 7 or 8 days after larval-pupal ecdysis as did the controls or the pupae treated with *Bombyx* Sephadex G–50

fractions. However, the pupae emerged one day earlier than the controls (day 6 or 7) when they were treated with one of the Sephadex G-50 fractions containing *Polygonia* materials of a larger molecular weight than 15,000.

In addition, the fractions showing the highest SMPH- and the highest 4K-PTTH-activities were obtained at different acetonitril concentrations by reversed-phase HPLC, indicating that the factor showing SMPH-activity did not seem to be identical with the one showing 4K-PTTH-activity in both *P. c-aureum* and *B. mori.* However, several microheterogenieties were shown to be present in the molecules of *Bombyx* 4K-PTTH (bombixin) [7]. We have left open the interesting question of whether or not one kind of 4K-PTTH molecule showing lower activity than the others produces summer morphs in *P. c-aureum.* The question may be solved during a course of further study concerning the roles SMPH plays in the development of seasonal morphs.

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REFERENCES

1 Fukuda, S. and Endo, K. (1966) Hormonal control of the development of seasonal morphs in the butterfly,*Polygonia c-aureum* L. Proc. Japan Acad., **42**: 1082–1087.

2 Hidaka, T. and Takahashi, H. (1967) Temperature condition and maternal effect as modifying factor in the photoperiodic control of seasonal forms in *Polygonia c-aureum* (Lepidoptera, Nymphalidae). Annot. Zool. Japon. **40**: 200–204.

3 Endo, K. (1972) Activation of corpora allata in relation to ovarian maturation in the seasonal forms of the butterfly, *Polygonia c-aureum* L. Dev. Growth Differ., **14**: 263–274.

4 Endo, K. (1984) Neuroendocrine regulation of the development of seasonal forms of the Asian comma butterfly, *Polygonia c-aureum* L. Dev. Growth Differ., **26**: 217–222.

5 Endo, K., Masaki, T. and Kumagai, K. (1988) Neuroendocrine regulation of the development of seasonal morphs in the Asian comma butterfly, *Polygonia c-aureum* L.: Difference in activity of summer-morph-producing hormone from brain-extracts of the long-day and short-day pupae. Zool. Sci., **5**: 145–152.

6 Ishizaki, H. and Ichikawa, M. (1967) Purification of the brain hormone of the silkworm, *Bombyx mori.* Biol. Bull., **133**: 355–368.

7 Ishizaki, H., Suzuki, A., Isogai, A., Nagasawa, H. and Tamura, S. (1977) Enzymatic and chemical inactivation of partially purified prothoracicotropic (brain) hormone of the silkworm, *Bombyx mori.* J. Insect Physiol., **23**: 1219–1222.

8 Suzuki, A., Matsumoto, S., Ogura, N., Isogai, A. and Tamura, S. (1976) Extraction and partial purification of the hormone inducing cuticular melanization in armyworm larvae. Agric. Biol. Chem., **40**: 2307–2309.

9 Sonobe, H. and Ohnishi, E. (1971) Silkworm *Bombyx mori* L.: nature of diapause factor. Science, **174**: 835–838.

10 Endo, K. and Funatsu, S. (1985) Hormonal control of seasonal morph determination in the swallowtail butterfly, *Papilio xuthus* L. (Lepidoptera: Papilionidae). J. Insect Physiol., **31**: 669–674.

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Effects of Water Temperature and Photoperiod on the Beginning of Spawning Season in the Orange-red Type Medaka[1]

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ABSTRACT—Rearing experiments were conducted to study effects of photoperiod and water temperature on the beginning of spawning season in the orange-red type medaka, *Oryzias latipes*. Fish, cultured in Tokyo and ready for rapid gonadal growth in spring, was transferred to 7 conditions (16°C 14L, 14°C 14L, 14°C 12L, 14°C 10L, 12°C 14L, 10°C 14L, 8°C 14L), and reared for 4 to 8 weeks. Changes in gonadosomatic index (GSI) and gonadal histology were examined. Groups at 14 and 16 °C showed rapid GSI increase irrespective of photoperiod. Active yolk globule accumulation and spermatogenesis began, and spawning was observed in these groups. After 8 weeks, many regressive oocytes were noticeable in 14°C groups. In groups at temperature below 14°C, GSIs of both sexes remained low and spawning was not observable. But yolk globule accumulation proceeded slowly in 10 and 12°C groups. When medaka in early spring were transferred to 4 conditions (16°C 10L, 8L, 6L, 4L), and reared for 4 weeks, they could mature and spawn in all experimental regimes, which eliminates possibility for photoperiodic response in spring. From these results, it is clear that temperature rise in spring only is responsible for the beginning of spawning season in medaka cultured around Tokyo.

INTRODUCTION

Most teleost fishes have their own annual reproductive cycles. Though some fishes seem to have endogenous reproductive rhythm [1], the annual reproductive cycle is generally believed to depend upon seasonal changes in environmental factors: photoperiod and water temperature in temperate zone, and rainfall in tropical zone [1–6].

Medaka, *Oryzias latipes*, is a small freshwater teleost native to Japan and her adjacent areas [7]. Two types of medaka, namely the wild and the orange-red types [8], are well known. Their annual reproductive cycles have already been investigated [9–12], and the spawning season is understood to extend usually from mid-April to early September.

There have been several works concerning environmental effects on the beginning of spawning season in medaka, with attention engaged by effects of photoperiod and/or water temperature [10, 13–19]. But obtained results did not always coincide with one another. As for the orange-red type medaka, in additions, effects of photoperiod in combination with water temparature have not been clearly shown yet, warranting further investigations.

This study was conducted to clarify environmental factors responsible for the beginning of spawning season in the orange-red type medaka. Rapid and remarkable gonadal growth, i.e. commencement of yolk globule accumulation in ovaries and retrieval of meiosis in testes, is leading and inevitable process for the beginning of spawning [9, 10]. Efforts were made to reveal environmental factors which cause this rapid gonadal growth. Thus medaka in early spring, which had been reared under natural condition and was ready for the rapid gonadal growth, was used as material. Photoperiod and temperature regimes were selected to be comparable to, or not too far from, natural environmental changes in spring.

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MATERIALS AND METHODS

Two experiments were conducted in 1980 (Experiment 1) and 1981 (Experiment 2). The orange-red type medaka *Oryzias latipes*, born in the preceding breeding season, were obtained from a hatchery in Edogawa-ku, Tokyo, kept in a pen (area 14 m^2, depth 40 cm) placed in a circulating outdoor tank (area 14 m^2, maximum depth 50 cm) at least for 4 months till experiments, and fed tubifex (freshwater oligochaetes) to satiation.

During experiments, water temperature in the outdoor tank ranged from 6 to 16°C, and the daylength [20] increased from 11.3 to 13.3L.

Experiment 1 was started on February 27 and ended on April 24. Fish ranging from 21 to 31 mm in standard length were transferred to following 7 conditions: 8°C 14L (14 hours of light/day), 10°C 14L, 12°C 14L, 14°C 14L, 14°C 12L, 14°C 10L, 16°C 14L. The three 14°C groups were reared for 8 weeks, and the others for 4 weeks. Twenty and 30 fish of each sex were used for 8°C, 10°C, 12°C or 16°C regime and three 14°C regimes, respectively. Part of fish in each group was taken on March 13 and 27, and the rest at the end.

Experiment 2 was started on March 6 and ended on April 7. Thirty fish of each sex, with standard length of 20 to 30 mm, were reared under 16°C 4L, 16°C 6L, 16°C 8L or 16°C 10L. Part of the fish were sampled halfway also in this experiment.

Each experimental group was kept in a 50 to 60 liter, light proof aquarium with circulation and some water plants. Photoperiod was controlled with a 10 W fluorescent lamp connected to a timer, and water temperature with a thermostat and heater. Fish were fed with tubifex *ad lib.*

After taken up, fish were anesthetized with tricaine methane sulfonate. The whole body with opened abdominal cavity was fixed in Holland's solution-sublimate in Experiment 1 and in Bouin's solution in Experiment 2. Standard length, body weight and gonad weight were measured while in 70% alcohol. GSI (gonad weight $\times$ 100/body weight) was calculated and gonads were embedded in paraffin by ordinary method. Ovarian sections in 10 μm were stained with AZAN and testicular sections in 5 μm with Mayer's hematoxylin and eosin.

RESULTS

Experiment 1

Figure 1 shows the changes in GSI under experimental regimes. Average GSI of initial controls was around 2.5% in female and 0.5% in male. The ovary contained oocytes up to yolk vesicle stage. The testis had spermatogonia, primary spermatocytes and residual sperm.

Females exposed to higher temperature (14 or 16°C) had rapid increases of GSI, reaching 6 to 9% in the fourth week, irrespective of photoperiod. The beginning of yolk globule accumulation was observed in the second week, and fully matured oocytes were noticeable in the fourth week. First spawning was observed in 14°C 12L group 25 days after the commencement, followed by spawning in the other groups at higher temperatures. GSIs of three groups at 14°C kept high till the eighth week, but ovaries of these groups contained many regressive oocytes at the end of the experiment.

Females exposed to lower temperatures (12, 10 or 8°C) had little increase of GSI in spite of long daylength. GSIs of 10°C 14L and 12°C 14L groups were 3 to 4% in the fourth week. Their ovarian histology in the second week was unchanged, but in the fourth week early yolk globule stage oocytes were observed. GSI and ovarian histology of 8°C 14L group in the fourth week were equal to those of initial control. No spawning was observed in these groups.

GSIs of males exposed to higher temperatures showed rapid increase, reaching 1.0 to 1.2% in the fourth week, and three groups at 14°C kept that value till the eighth week. Increase of spermatogonia and primary spermatocytes was observed in the second week, leading to active spermatogenesis shown by meioses of spermatocytes in the fourth and eighth weeks.

Males exposed to lower temperature showed a little increase of GSI, reaching 0.6 to 0.8% in the fourth week. But active spermatogenesis was not observed.

Experiment 2

Results of Experiment 1 indicated importance of rising temperature in the beginning of spawning

season in medaka, daylength changes having little effect on gonads. Experiment 2 was conducted to examine the possibility of photoperiodic response in gonadal development.

Figure 2 shows the changes of GSI under experimental regimes. Average GSI of initial controls was around 2.0% in female and 0.5% in male. Gonadal histology of initial controls was much the

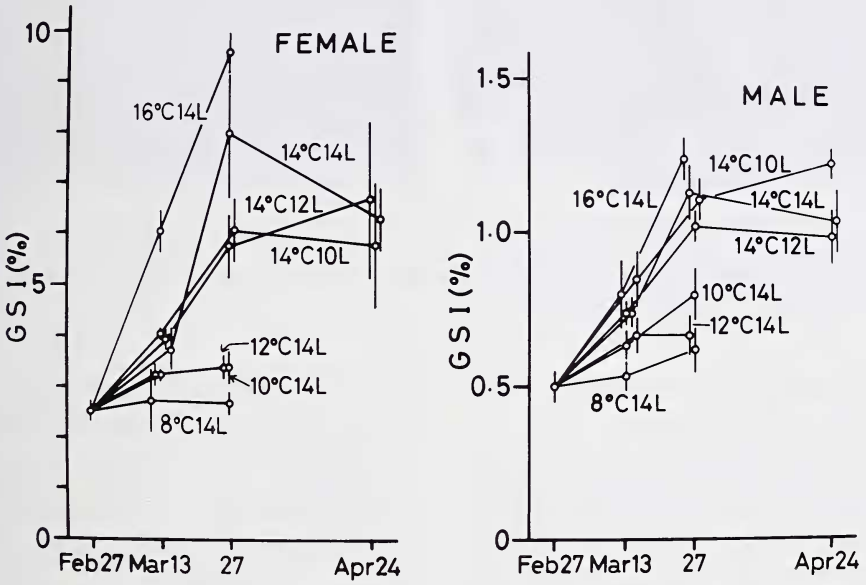

FIG. 1. Changes in the gonadosomatic index (GSI) of the orange-red type medaka reared under various temperature-photoperiod regimes in Exp. 1.

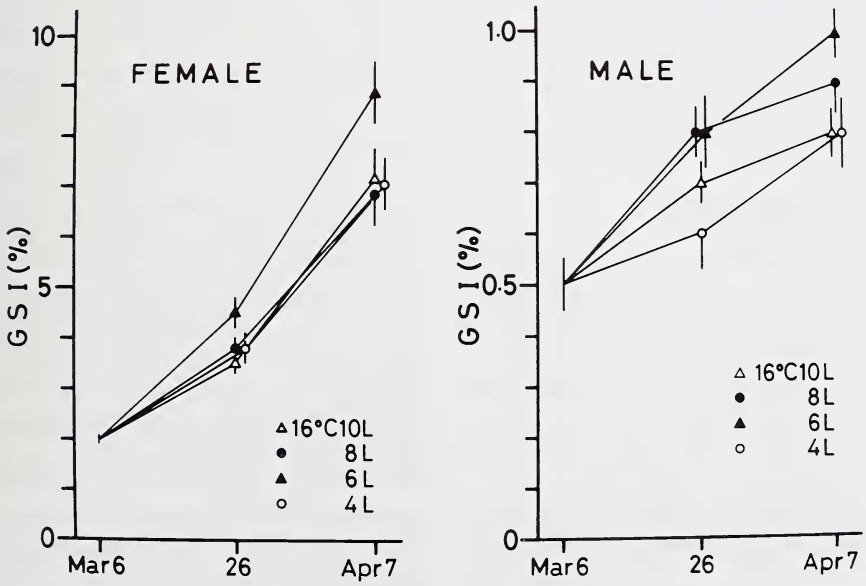

FIG. 2. Changes in the gonadosomatic index (GSI) of the orange-red type medaka reared under short day regimes in Exp. 2.